

## METHOD FOR HIGH-THROUGHPUT SELECTION OF INTERACTING MOLECULES

The present invention relates to a method for the selection of at least one member of a number of specifically interacting molecules, said method being carried out in (a) container(s), preferably representing an arrayed form, e.g. in (a) microtiter plate(s), using an automated device comprising a magnetic particle processor. In another embodiment, the present invention relates to a method for the production of a pharmaceutical composition comprising the steps of the method of the present invention and further the step of formulating at least one of said specifically interacting molecules selected and/or characterized by the above method or a functionally and/or structurally equivalent derivative thereof in a pharmaceutically acceptable form.

Cellular functions are controlled by networked expression of gene catalogues. Functional network analysis requires parallel handling of large numbers of gene products and selection and characterization of interacting molecules. For studying protein interactions, two classical library-based approaches are known, an in vitro method, the so-called yeast Two-Hybrid-System (Mendelsohn & Brent, Science 284, 1999, 1948-1950) and in vitro methods, e.g. phage display. The yeast Two-Hybrid-System has the following disadvantages: (i) transformation efficiency in yeast is low; (ii) protein interactions take place in the milieu of the yeast nucleus and, therefore, interaction parameters can not be controlled; and (iii) only protein-protein interactions are possible to be investigated.

In vitro (e.g. phage surface) display enables the construction of large recombinant peptide and protein libraries for the selection of interacting molecules. The basic concept is a physical link of the phenotype, expressed as gene product (e.g. displayed on the phage surface) to its coding genetic information (e.g. integrated into the phage

genome). This allows to survey large libraries of organisms (e.g. phage, viruses, bacteria, eukaryotic cells) and/or organelles (e.g. ribosome) and/or soluble molecules (e.g. nucleic acids, protein-nucleic acid hybrids) for the presence of specific molecules using the discriminative power of affinity purification. The selection procedure involves the enrichment of a specific first molecule by binding to an immobilized second (target) molecule. First molecules are enriched by selection (binding and elution) on the target molecule. As a consequence of the physical linkage between genotype and phenotype, sequencing the DNA of the encoding first molecule can readily elucidate the amino acid sequence of the selected gene product.

Peptide libraries were the first libraries to be displayed on phage (Smith, 1985). In the meantime, a wide variety of different peptide libraries were made, with different degrees of randomness and special means of recombination (e.g., Fisch *et al.*, 1996). Peptide libraries are especially useful for mapping interacting parts of proteins (e.g., domains or epitopes). They are also a first step towards the production of small molecules simulating protein actions.

Recombinant immunoglobulin gene libraries cloned in phage or phagemid vectors are an in vitro simulation of antibody repertoires and allow the production of antibodies without immunisation and without the use of animals (reviewed in Winter *et al.*, 1994). Human antibodies against large numbers of different antigens, including human proteins, can be produced by phage selection of single-chain Fv (scFv; Nissim *et al.*, 1994) or Fab fragments (Griffiths *et al.*, 1994). Those antibodies should be particularly valuable as therapeutic agents because the patient's immune system will not recognise them as foreign because they are completely human. Besides antibodies, also enzymes, enzyme inhibitors, receptors, hormones, lymphokines and DNA-binding molecules have been target molecules displayed on filamentous phage. The wide range of possible applications clearly demonstrates the high potential of linking genotype and phenotype as a tool for the development of new molecules.

Although phage display was used extensively for the selection of peptides and antibodies, it had its limitations when it came to the expression of unknown sequences from cDNA libraries. As many of these sequences contain stop codons in their 3' untranslated regions, one cannot directly fuse these sequences to the N-terminus of a

phage coat protein. To overcome this problem, a specialised cloning and expression system has been developed that allows the display of functional cDNA expression products on the surface of filamentous bacteriophage (Crameri & Suter, 1993; Crameri & Blaser, 1995). This system exploits the high-affinity interaction of the Jun and Fos leucine zippers. Gene jun is expressed from a lacZ promoter as a fusion protein with the phage coat protein III. Using a second lacZ promoter of the phagemid pJuFo, gene fos is co-expressed as an N-terminal fusion peptide with the cDNA library gene products, so that the resulting Fos-fusion proteins could become associated with the Jun-decorated phage particles. To avoid inter-phage exchange of fos-cDNA fusion products, cysteines were engineered at the N- and C-termini of each of the leucine zippers, providing a covalent link of the cDNA gene products to the genetic instructions required for their production.

The physical link between phenotype and genotype in phage display allows selective isolation and amplification of a particular phage encoding a desired gene product from pools of millions of phage (Kay *et al.*, 1996). Selection is accomplished by interaction between the displayed gene product and a ligand immobilized on a solid phase. The selected phage are amplified by infection of *E. coli* cells which, after helper rescue, produce large numbers of new phage. Successive rounds of phage selection and amplification allow selective enrichment of phage displaying gene products with affinity for a desired ligand.

In the prior art, target molecules have been immobilized on plastic surfaces, mainly immunotubes or microtiter plate wells (Harrison *et al.*, 1996). Also, magnetic particles have been used for phage display selection (Hawkins *et al.*, 1992, Griffiths *et al.*, 1994, Low *et al.*, 1996, Schier *et al.*, 1996a; Schier *et al.*, 1996b, McConnell *et al.* 1998, McConnell *et al.*, 1999, Kirkham *et al.*, 1999). However, these methods involve the manual handling of the samples and are cumbersome and time-consuming. Accordingly, these methods allow the simultaneous processing of only a very limited number of samples, i.e. they allow only the identification and characterization of one or, at most, a few pairs of interacting molecules per one selection procedure. Moreover, they are difficult to standardize in terms of precisely reproducible conditions.

Thus, the technical problem underlying the present invention was to provide a method that allows the reliable, simultaneous and time-saving high-throughput selection of various members of pairs of interacting molecules.

The solution to the above technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the present invention relates to a method for the selection of at least one member of a number of specifically interacting molecules, said method comprising as the first step involving the contact of said interacting molecules:

- (a) contacting a first molecule with a second molecule affixed to a magnetic particle under conditions that allow a specific interaction between said first and second molecule to occur;

and further the steps of:

- (b) subjecting the product obtained in step (a) to at least one washing step;
- (c) determining whether a specific interaction between said first and second molecule had occurred; and, if said specific interaction had occurred,
- (d) providing said first and/or second molecule selected by steps (a) to (c),

wherein steps (a), (b) and (c) are carried out in (a) container(s), preferably representing an arrayed form, e.g. in (a) microtiter plate(s), using an automated device comprising a magnetic particle processor (Fig. 1).

The method of the present invention shows several unexpected advantages in terms of sensitivity, control and automation: First, the number of magnetic particles can be scaled down compared to the manual techniques (e.g. 10-fold to 2  $\mu$ l or  $1.34 \times 10^6$  Dynabeads M-280 Streptavidin, Dynal). This causes much less unspecific background binding resulting in a distinct reduction of false positive results.

Second, all washing and incubation conditions can be reproducibly customized. Most importantly, it is envisaged in accordance with the present invention that washing speeds are adjusted to cause different stringencies of selection. This will enable the predictable selection of interacting molecules with different binding affinities. The

washing step which may be repeated at least once in each round of selection is designed to remove first molecules that did not specifically interact with/bind to said second molecules. Appropriate washing conditions can be taken from the appended examples or devised by the person skilled in the art without undue burden.

Third, it is envisaged in accordance with the present invention that the steps of (i) the provision, preferably the recombinant production of said second molecule (that may be a member of one library), (ii) affixing said second molecule to magnetic particles, (iii) optionally blocking of free binding sites on the magnetic particles (to which no second molecules had been affixed), (iv) contacting said first molecule (that may be a member of another library) with said second molecule, (v) washing steps, and (vi) the determination whether a specific interaction between said first and second molecule had occurred, are carried out in (a) container(s) preferably representing an arrayed form, e.g. in (a) microtiter plate(s) or container(s) comprising tubes in an arrayed form, wherein each step is preferably performed in (a) different container(s). The magnetic particle processor comprised in said automated device is used to transfer the magnetic particles between wells of microtiter plates prefilled with the corresponding solutions by capturing the magnetic particles in a first well and releasing the same in a second well of a different microtiter plate, the position of said second well corresponding to the position of said first well. This allows high-throughput selection of interacting molecules as large numbers of, e.g., library clones can be handled in parallel, and the selection of interacting molecules from, e.g., two libraries can be used to create interaction catalogues.

In this regard it is to be understood that a "selection round" comprises steps (a) to (c). Accordingly, the phrase "first step involving the contact of said interacting molecules" denotes the contacting step of the first selection round as compared to second, third, etc. steps involving the contact of said interacting molecules of potential further selection rounds that may be performed subsequently to the first selection round (see below).

A preferred mode of the selection at high-throughput of the invention comprises the following steps: interacting molecules (e.g., anti-protein scFv antibodies) are selected from molecular libraries by a combination of phage display and magnetic bead

technology. Proteins expressed from arrayed cDNA libraries are bound to magnetic beads via a suitable molecular tag (e.g., His<sub>6</sub> or Biotin). Phage displaying specifically interacting molecules are then fished from a library by binding to their interaction partners attached to the beads. This selection involves a sequence of binding and washing steps and was adapted to high throughput using a magnetic particle processor (Labsystems, Helsinki, Finland). Selected molecules are then tested for specificity also employing the magnetic particle processor.

Said first and second molecule may be members of libraries, e.g., an antibody and an antigen library, respectively, i.e. two different libraries. Alternatively, said first and second molecule may be members of the same library. Also comprised by the present invention are embodiments, wherein one molecule (i.e. the first or the second molecule) is a member of a library whereas the other molecule is a compound or a variety of compounds of predetermined specificity. Other options to employ first and second molecules from still different origins or combinations of origins are within the skills of the person skilled in the art.

In a preferred embodiment of the method of the present invention said first and/or second molecule is an organic molecule and/or a mixture of organic and/or inorganic molecules.

In another preferred embodiment, said first and/or second molecule is a hapten.

In a more preferred embodiment of the method of the present invention said first and/or second molecule is a cDNA expression product, and/or a (poly)peptide, and/or a nucleic acid, and/or a lipid, and/or a sugar, and/or a steroid, and/or a hybrid of said molecules.

In a most preferred embodiment said cDNA expression product is an antibody or a fragment or a derivative thereof, an enzyme or a fragment thereof, a surface protein or a fragment thereof, or a nucleic acid-binding protein or a fragment thereof.

Derivatives and fragments of antibodies are well known in the art and comprise, e.g.,  $F(ab')_2$ , Fab, Fv or single chain Fv antibody fragments (see, e.g., Harlow and Lane, "Antibodies, a laboratory manual", CHS Press, 1988, Cold Spring Harbor, N.Y.).

In another preferred embodiment of the method of the present invention said first molecule is a (poly)peptide presented on the surface of organisms (e.g. phage, viruses, bacteria, eukaryotic cells) and/or organelles (e.g. ribosome) and/or soluble molecules (e.g. nucleic acids, protein-nucleic acid hybrids) and the method further comprises after step (b) and prior to step (c) the step of:

(b') amplifying a (poly)peptide specifically interacting with said second molecule, wherein step (b') is carried out in (a) container(s) preferably representing an arrayed form, e.g. in (a) microtiter plate(s).

In one embodiment of the present invention relating to bacteriophage surface display, the magnetic particle processor may be used to transfer the magnetic particles (to which said bacteriophage is bound via said (poly)peptide specifically interacting with said second molecule) to bacterial culture(s). In another embodiment, the automated device further comprises a shaking device that may be used for shaking the bacterial culture(s) during amplification of said bacteriophages.

It could be demonstrated in accordance with the present invention that after binding of the polypeptide presented on the surface of a bacteriophage to said second (target) molecule affixed to a magnetic particle, said bacteriophage retains its infectivity. Surprisingly, it is possible for the bacteriophage amplification step to infect bacteria directly after a specific interaction has occurred without the need of prior detachment of bound bacteriophage from the magnetic particle.

In a more preferred embodiment of the method of the present invention prior to step (a) said library of first molecules (library 1) is preabsorbed with unloaded magnetic particles and/or molecules competitive (cross-reactive) to second molecules (target, library 2).

For example, if a library of phages is used for performing the method of the invention, this step ensures that phages unspecifically interacting with the magnetic particles are

removed from the phage mixture and only bacteriophages displaying a specifically interacting molecule are selected via this specific interaction. In other words, this step may be performed to further reduce the number of false positive clones.

In an additional more preferred embodiment the method of the present invention further comprises after step (c) and prior to step (d) the step of:

(c') repeating steps (a), (b) and (c) and, optionally, step (b') at least once.

In a most preferred embodiment of the method of the present invention steps (c) and (c') are performed in parallel.

As mentioned above, steps (c) and (c') of the method of the present invention are each carried out in microtiter plates. This advantageously allows the simultaneous performance of steps (c) and (c') in different microtiter plates which further reduces the time required for practicing the method of the present invention.

In a further preferred embodiment of the method of the present invention said number of specifically interacting molecules is a pair of interacting molecules.

In another preferred embodiment of the method of the present invention said number of specifically interacting molecules are three or more interacting molecules.

In yet a further preferred embodiment, the method of the present invention further comprises the step of characterizing said first and/or second molecule and/or the corresponding genetic information.

Methods for the characterization of genetic information, i.e. nucleic acids, and proteinaceous material are well known in the art and include, e.g., nucleic acid sequencing, southern-, northern-, and colony hybridization, primer extension analysis, RNase protection assay, gel shift analysis, western-blotting, ELISA, immunoprecipitation assay, indirect immunofluorescence analysis, and FACS (see, e.g., Sambrook et al., "Molecular cloning - a laboratory manual", Cold Spring Harbor Laboratory (1989) N.Y., Ausubel et al., "Current protocols in molecular biology", Green



Publishing Associates and Wiley Interscience, N.Y. (1989), and Harlow and Lane, loc. cit.).

In another preferred embodiment, said second molecule (target) is affixed to said magnetic particle via an affinity tag (e.g. a metal-chelating tag, an epitope tag, an enzyme binding domain, calmodulin, biotin, Strep-tag, protein A, protein G or protein L) (Fig. 2).

The use of one of the above-mentioned compounds advantageously ensures that said second molecule is affixed to said magnetic particle in a controlled manner and in a predictable orientation, thereby minimally affecting the three-dimensional structure of said second molecule and, consequently, the interacting capacities. Moreover, the use of the above-mentioned compounds allows the direct loading of magnetic particles with second molecules from protein mixtures like, e.g., crude extracts or cell lysates. This is particularly important for high-throughput selection since purification of large numbers of different second molecules is not necessary. However, although the use of the above-mentioned compounds is preferred, the present invention also encompasses the unspecific adsorption of second molecules, e.g., to magnetic particles coated with a plastic surface and/or the covalent binding of second molecules, e.g., via functional groups such as  $\text{NH}_2$ -,  $\text{COOH}$ -,  $\text{SH}$ -groups. These modes of loading may be used especially if partial denaturation and destruction of, e.g., epitopes does not affect the overall efficiency of the method of the present invention.

Moreover, it has been found out in accordance with the present invention that for affixing said second molecule to said magnetic particle preferably saturating concentrations of said second molecule are used so that virtually all free binding sites on said magnetic particle are bound by said second molecule. However, the method of the present invention also encompasses the work with sub-saturating concentrations of said second molecule.

In a most preferred embodiment, said metal-chelating tag is a His-tag, and/or said epitope tag is an HA-tag, a c-myc-tag, a VSV-G-tag, an  $\alpha$ -tubulin-tag, a B-tag, an E-tag, FLAG, a His-tag, an HSV-tag, a Pk-tag, a protein C-tag, a T7-tag, EpiTag™, a V5-

tag or an S-tag, and/or said enzyme binding domain is cellulose binding domain, barnase or maltose binding protein.

In an additional preferred embodiment of the method of the present invention step (c) is effected by immunological means.

In a more preferred embodiment of the method of the present invention step (c) is effected by ELISA, RIA, western/colony blotting, FACS or immunohistochemistry.

In another more preferred embodiment of the method of the present invention step (c) is effected in (micro-)array format, preferably on a membrane and/or filter and/or a glass slide and/or in a microtiter plate.

The present invention also relates to a method for the production of a pharmaceutical composition comprising the steps of the method of the present invention and further the step of formulating said first and/or second molecule selected and/or characterized by the method described hereinabove or a functionally and/or structurally equivalent derivative thereof in a pharmaceutically acceptable form.

The pharmaceutical composition of the present invention may comprise a pharmaceutically acceptable carrier and/or diluent. Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by well known conventional methods. These pharmaceutical compositions can be administered to the subject at a suitable dose. Administration of the suitable compositions may be effected by different ways, e.g., by intravenous, intraperitoneal, subcutaneous, intramuscular, topical, intradermal, intranasal or intrabronchial administration. The dosage regimen will be determined by the attending physician and clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general

health, and other drugs being administered concurrently. A typical dose can be, for example, in the range of 0.001 to 1000  $\mu\text{g}$  (or of nucleic acid for expression or for inhibition of expression in this range); however, doses below or above this exemplary range are envisioned, especially considering the aforementioned factors. Generally, the regimen as a regular administration of the pharmaceutical composition should be in the range of 1  $\mu\text{g}$  to 10 mg units per day. If the regimen is a continuous infusion, it should also be in the range of 1  $\mu\text{g}$  to 10 mg units per kilogram of body weight per minute, respectively. Progress can be monitored by periodic assessment. Dosages will vary but a preferred dosage for intravenous administration of DNA is from approximately  $10^6$  to  $10^{12}$  copies of the DNA molecule. The compositions of the invention may be administered locally or systemically. Administration will generally be parenterally, e.g., intravenously; DNA may also be administered directly to the target site, e.g., by biolistic delivery to an internal or external target site or by catheter to a site in an artery. Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like. Furthermore, the pharmaceutical composition of the invention may comprise further agents such as interleukins or interferons depending on the intended use of the pharmaceutical composition.

The term "functionally and/or structurally equivalent derivative" as used in accordance with the present invention denotes molecules modified, e.g., by deletion, addition and/or substitution of certain parts thereof but essentially maintaining their capacity of specifically interacting with said first or second molecule selected by the method of the present invention. Also encompassed by this term are molecules that have been

modified in order, e.g., to increase their half-lives in a subject to which they have been administered, to increase the rate of their uptake, to increase their affinity to their interacting counterparts or to increase the excretion rate of the corresponding metabolized end products. With regard to nucleic acids, such molecules may be peptide nucleic acids or nucleic acids comprising, e.g., methylphosphonate- or phosphorothioate-bonds instead of phosphodiester-bonds. Methods for the synthesis of derivatives that, e.g., show the same three-dimensional structure than the originally identified molecule are known in the art and include, e.g., peptidomimetics (see, e.g., Hruby, V.J. et al., Biopolymers 43(3) (1997), 219-66; Bohm, H. J., J. Comput. Aided Mol. Des. 10(4) (1996), 265-272; Wiley, R.A. & Rich, D. H., Med. Res. Rev. 13(3) (1993), 327-384; al-Obeidi, F. et al., Mol. Biotechnol. 9(3) (1998), 205-223; Beeley, N., Trends Biotechnol. 12(6) (1994), 213-6).

Further preferred is to use the compound provided in accordance with the present invention as lead compound for providing downstream developments, in accordance with methods presently employed in the art.

In addition, the invention relates to pharmaceutical compositions comprising at least one of the selected interacting molecules or of derivatives as defined above, optionally in combination with a pharmaceutically acceptable carrier and/or diluent.

The documents cited herein are herewith incorporated by reference.

The figures show:

**Figure 1:** High-throughput selection of binding partners. Magnetic particles are transferred between wells of microtitre plates, incubated and washed using an automated magnetic particle processor. Molecules of the arrayed Library 2 (targets) are tag-bound to magnetic particles which are washed, blocked and incubated with Library 1 being, e.g., a phage display library. After washing away background phage and incubation

with bacteria and helper phage, an enriched and amplified Library 1 enters the next round of selection against the same Library 2 molecules for further enrichment.

**Figure 2:** Example for the selection of interacting molecules. Selection from phage display libraries of, e.g. human scFv antibody fragments recognising targets (e.g. specific expression products of a human cDNA library) tag-bound to magnetic particles.

**Figure 3:** Automated magnetic particle processor (Labsystems, Helsinki, Finland) in action. Left: rod-shaped magnets and plastic caps separated, magnetic particles in solution in microtitre wells; top right: magnets in plastic caps, collection of magnetic particles to plastic caps; bottom right: transfer of magnetic particles to new pre-filled microtitre wells.

**Figure 4:** Saturation ELISA for assessment of optimal concentrations of protein targets (e.g. bGAPDH) for loading of magnetic particles.

**Figure 5:** Polyclonal mixtures of phage representing the unselected (rounds) library 1 and the results of every round of selection screened for binding partners to the protein target used for this selection (e.g. UBI8) using magnetic particle ELISA; PTM negative control.

**Figure 6:** Monoclonal phage (e.g. anti-UBI8) rescreened by magnetic particle phage ELISA for binding to the same protein target (e.g. UBI8); PTM negative control.

The examples illustrate the invention.

**Example 1:** Automated magnetic particle-handling

An automated device (Fig. 3, Labsystems) was used for washing and incubation of magnetic particles. 96-well microtitre plates (e.g. CliniPlate 200, Labsystems) were pre-filled with solutions (200 µl), and magnetic particles were transferred between wells by capture to and release from rod-shaped magnets covered with plastic caps. Ni-NTA Silica Beads (Qiagen, Hilden, Germany) or Dynabeads M-280 Streptavidin (Dynal, Oslo, Norway) were used for binding of His<sub>6</sub>- or biotin-tagged proteins, respectively.

**Example 2:** Large-scale target production and purification

Protein targets were expressed in *E. coli* (strain SCS1) liquid cultures. 200 ml 2xTY medium (16 g/l Bacto-tryptone, 10 g/l yeast extract, 5 g/l NaCl, pH 7.0) containing 100 µg/ml ampicillin were inoculated with 2 ml of an overnight culture and shaken at 37°C until an OD<sub>600</sub> of 0.8 was reached. Isopropyl-b-D-thiogalactopyranosid (IPTG) was added to a final concentration of 1 mM. The culture was shaken for 4-6 h at 30 or 37°C. Cells were harvested by centrifugation at 2,100 g for 10 min, resuspended in 5 ml Lysis Buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.3 M NaCl, 10 mM Imidazole, 0.1 mM PMSF, pH 8.0) containing 0.25 mg/ml lysozyme, 10 µg/ml DNase and 10 µg/ml RNase and incubated on ice for 30 min. DNA was sheared with an ultrasonic homogeniser (Sonifier 250, Branson Ultrasonics, Danbury, USA) for 3 x 1 min at 50% power on ice. The lysate was cleared by centrifugation at 10,000 g for 30 min. Ni-NTA agarose (Qiagen) was added and mixed by shaking at 4°C for 1 h. The mixture was poured into a column which was subsequently washed with ten bed volumes of Lysis Buffer containing 20 mM imidazole. Protein was eluted in Lysis Buffer containing 250 mM imidazole and was dialysed against Phosphate-Buffered Saline (PBS, 10 mM Phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4) at 4°C overnight.

Proteins were either expressed as fusion proteins with a His<sub>6</sub>- and/or biotin-tag attached (Lueking *et al.*, 1999) or were biotinylated in vitro using ImmunoPure NHS-SS-Biotin (Pierce, Rockford USA) and the efficiency of biotinylation was determined by ImmunoPure HABA (Pierce).

**Example 3:** High-throughput small-scale target production (native conditions)

High-throughput small-scale protein expression and purification was modified according to (Lueking *et al.*, 1999). Briefly, proteins were expressed from selected clones of the arrayed human fetal brain cDNA expression library hEx1 (Büssow *et al.*, 1998). This library was directionally cloned in pQE-30NST for IPTG-inducible expression of His<sub>6</sub>-tagged fusion proteins. 96-well microtitre plates (e.g. CliniPlate 200, Labsystems) were filled with 180 µl 2xTY medium supplemented with 100 µg/ml ampicillin. Cultures were inoculated with 20 µl *E. coli* SCS1 cells from overnight cultures. After growth at 37°C with vigorous shaking until an OD<sub>600</sub> of 0.2 was reached, IPTG was added to a final concentration of 1 mM. Cells were grown for 4-6 h at 30 or 37°C, harvested by centrifugation at 6,000 g for 10 min, washed by resuspension in Lysis Buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.3 M NaCl, 10 mM Imidazole, 0.1 mM PMSF, pH 8.0) containing 0.25 mg/ml lysozyme, 10 µg/ml DNase and 10 µg/ml RNase and incubated on ice for 1 h.

**Example 4:** High-throughput small-scale target production (denaturing conditions)

High-throughput small-scale protein expression and purification was described (Lueking *et al.*, 1999). Briefly, proteins were expressed from selected clones of the arrayed human fetal brain cDNA expression library hEx1 (Büssow *et al.*, 1998), directionally cloned in pQE-30NST for IPTG-inducible expression of His<sub>6</sub>-tagged fusion proteins. 96-well

microtitre plates with 2 ml cavities (StoreBlock, Zinsser) were filled with 100 µl SB medium, supplemented with 100 µg/ml ampicillin and 15 µl/ml kanamycin. Cultures were inoculated with *E. coli* SCS1 cells from 384-well library plates (Genetix, Christchurch, U.K.) that had been stored at -80°C. For inoculation, replicating devices carrying 96 steel pins (length 6 cm) were used. After overnight growth at 37°C with vigorous shaking, 900 µl of prewarmed medium were added to the cultures, and incubation was continued for 1 h. For induction of protein expression, IPTG was added to a final concentration of 1 mM. All following steps, including centrifugations, were also done in 96-well format. Cells were harvested by centrifugation at 1,900 g (3,400 rpm) for 10 min, washed by resuspension in Phosphate Buffer, centrifuged for 5 min and lysed by resuspension in 150 µl Buffer A (6 M Guanidinium-HCl, 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, 0.01 M Tris-HCl, pH 8.0). Bacterial debris was pelleted by centrifugation at 4,000 rpm for 15 min. Supernatants were filtered through a 96-well filter plate containing a non-protein binding 0.65 µm pore size PVDF membrane (Durapore MADV N 65, Millipore, Bedford, USA) on a vacuum filtration manifold (Multiscreen, Millipore).

**Example 5: Magnetic particle loading**

Magnetic particles (10 µl or 250 µg Ni-NTA Silica Beads, Qiagen, or 20 µl or  $1.34 \times 10^7$  particles, Dynabeads M-280 Streptavidin, Dynal) were washed twice in PBST (PBS, 0.1 % Tween 20) and loaded with ligands as follows.

(a) Binding from lysates:

Magnetic particles were incubated for 1 h at RT in 200 µl total cell lysate. Examples were proteins like expression products of a human cDNA library as described (Lueking et al., 1999).

(b) Binding of purified targets:



Magnetic particles were incubated for 1 h at RT in 200 µl PBS containing 1% bovine serum albumin (BSA) and the equivalent of 5-10 µM purified targets, depending on the size of target. Examples were proteins, like human glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Swiss-Prot P04406), a C-terminal fragment (40.3 kd) of human heat shock protein 90 alpha (HSP90a, Swiss-Prot P07900), rat immunoglobulin heavy chain binding protein (BIP, Swiss-Prot P06761), tubulin alpha-1 chain (TUBa1, Swiss-Prot P04687), calcium-binding protein ERC-55 precursor (ERC55, Swiss-Prot Q14257), transcription elongation factor S-II (HS-II-T1, Swiss-Prot Q15560), transcription factor ETR101 (ETR101, Swiss-Prot Q03827), peptidyl-prolyl cis-trans isomerase A (EC5218, Swiss-Prot P05092) and Ubiquitin (UBIQ-HUMAN, SWISS-Prot P02248).

After target loading, magnetic particles were washed twice in PBST. Remaining free binding sites were blocked with PTM (PBS, 2% milk powder, 1 % Tween) for 1 h at RT.

#### **Example 6: Magnetic particle ELISA**

Magnetic particles (10 µl or 250 µg Ni-NTA Silica Beads, Qiagen, or 2 µl or  $1.34 \times 10^6$  particles, Dynabeads M-280 Streptavidin, Dyal) were incubated with primary and secondary antibodies diluted in PTM for 30 min at RT each and washed twice in PBST after antibody incubations. Secondary antibodies labelled with horseradish peroxidase (HRP) were detected using ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) solution (10 ml 50 mM Na<sub>3</sub> citrate, 10 ml 50 mM citric acid, 10 mg ABTS, 10 µl 30 % H<sub>2</sub>O<sub>2</sub>, pH 4.3) and measured as OD 405 nm using SpectraMAX 250 (Molecular Devices, Sunnyvale, USA).

#### **Example 7: Saturation ELISA**

Target dilutions (100 nM - 25  $\mu$ M) were prepared in 200  $\mu$ l PBS containing 1% BSA and incubated consecutively with two aliquots of magnetic particles (10  $\mu$ l or 250  $\mu$ g Ni-NTA Silica Beads, Qiagen, or 20  $\mu$ l or  $1.34 \times 10^7$  particles, Dynabeads M-280 Streptavidin, Dynal) for 1h at RT each. Magnetic particles were washed twice in PBST, blocked with PTM for 1 h at RT, incubated with primary and secondary antibodies diluted in PTM for 30 min at RT each and washed twice in PBST after antibody incubations. Secondary antibodies labelled with HRP were detected using ABTS solution and measured as OD 405 nm.

**Example 8:** Phage selection

The preparation of phage from bacterial glycerol stock of phage display libraries was described previously (Harrison *et al.*, 1996). Phage suspensions ( $10^{12}$  phage of unselected libraries or the MultiScreen flow-through after phage amplification between selection rounds) were equilibrated and preabsorbed by incubation with unloaded magnetic particles (10  $\mu$ l or 250  $\mu$ g Ni-NTA Silica Beads, Qiagen, or 25  $\mu$ l or  $1.68 \times 10^7$  particles, Dynabeads M-280 Streptavidin, Dynal) in 200  $\mu$ l PTM for 1h at RT.

Magnetic particles (10  $\mu$ l or 250  $\mu$ g Ni-NTA Silica Beads, Qiagen, or 20  $\mu$ l or  $1.34 \times 10^7$  particles, Dynabeads M-280 Streptavidin, Dynal) were loaded with targets and blocked as described above, incubated with preabsorbed phage for 1 h at RT and washed in PBST several times according to the number of the selection round. *E. coli* TG1 cells were incubated with the washed magnetic particles for 30 min at RT.

**Example 9:** Phage amplification between selection rounds

20  $\mu$ l 2xTY containing 10 x Glu-Amp (20% glucose, 1 mg/ml ampicillin) were added and cultures were shaken overnight at 37°C. 10  $\mu$ l of these

cultures were diluted into 200  $\mu$ l 2xTY containing 2% glucose, 100  $\mu$ g/ml ampicillin and shaken at 37°C until OD600 > 0.1 (SpectraMAX 250, Molecular Devices). 10  $\mu$ l M13-K07 helper phage ( $10^{12}$ /ml) were added, cultures were incubated for 30 min at RT and transferred to a Durapore 0.65  $\mu$  plate (Millipore). Cultures were sucked through on a MultiScreen vacuum device (Millipore). TG1 cells were resuspended in 200  $\mu$ l 2xTY containing 100  $\mu$ g/ml ampicillin and 60  $\mu$ g/ml kanamycin, transferred to a microtitre plate (e.g. CliniPlate 200, LabSystems) and vigorously shaken overnight at 30°C. Cultures were transferred to a Durapore 0.65  $\mu$  plate (Millipore) and sucked through on a MultiScreen vacuum device (Millipore). The flow-through was collected in a microtitre plate (e.g. CliniPlate 200, LabSystems) and was used as starting material (phage suspension) for the next round of selection.

#### Example 10: Magnetic particle phage ELISA

##### (a) Polyclonal ELISA

Phage were prepared from overnight cultures as described above. Magnetic particles (10  $\mu$ l or 250  $\mu$ g Ni-NTA Silica Beads, Qiagen, or 2  $\mu$ l or  $1.34 \times 10^6$  particles, Dynabeads M-280 Streptavidin, Dynal) were loaded with targets and blocked as described above, incubated with phage suspensions ( $10^{10}$ - $10^{11}$  phage) for 30 min at RT and washed twice in PBST. For detection, magnetic particles were incubated with anti-M13 HRP (1:5,000) in PTM for 30 min at RT, washed twice in PBST and incubated in ABTS solution which was measured at OD 405 nm.

##### (b) Monoclonal ELISA

The preparation of monoclonal phage was described previously (Harrison et al., 1996). Phage suspensions were divided into two aliquots, diluted 1:1 with PBS and incubated in parallel with magnetic particles either loaded with targets and blocked as described above or unloaded for 30 min at RT. Magnetic particles were washed twice in

PBST, incubated with anti-M13 HRP (1:5,000) in PTM for 30 min at RT, washed twice in PBST and incubated in ABTS solution which was measured at OD 405 nm.

**Example 11:** PCR and DNA sequencing

PCR and DNA sequencing of antibody genes was described previously (Walter & Tomlinson, 1996).

**Example 12:** BIAcore analysis

BIAcore analysis of antibody affinity was described previously (Hefta *et al.*, 1996).

**Example 13:** Specific tag-binding of targets to magnetic particles

His<sub>6</sub>- tagged proteins were bound to Ni-NTA Silica Beads (Qiagen), and biotin-tagged proteins were bound to Dynabeads M-280 Streptavidin (Dyna). This tag-binding is specific for the labelled molecules and ensures their proper orientation on the magnetic particles. In contrast, unspecific adsorption of proteins to plastic surfaces leads to partial denaturation and destruction of epitopes. Tag-binding also enables direct loading of magnetic particles with targets from protein mixtures like crude extracts or cell lysates. This is particularly important for high-throughput selection technology, avoiding purification of large numbers of different proteins or other targets.

**Example 14:** Optimisation of target concentration by Saturation ELISA

The concentration of target molecules on solid surfaces is a critical parameter for the selection of binding partner molecules. Optimal

concentrations of protein targets (e.g. bGAPDH) for loading of magnetic particles were assessed in saturation ELISA experiments (Fig. 4). For most proteins tested, concentrations of 5-10  $\mu$ M purified target (e.g. 200-400  $\mu$ g/ml bGAPDH) were found to cause saturation of 10  $\mu$ l (250  $\mu$ g) Ni-NTA Silica Beads (Qiagen) or 20  $\mu$ l ( $1.34 \times 10^7$  particles) Dynabeads M-280 Streptavidin (Dyna). These concentrations are in excess of saturating concentrations of other plastic surfaces (e.g. microtitre plates, Kala *et al.*, 1997) and confirm the high binding capacity of magnetic particles, reflecting their increased surface area. While it is advisable to work with saturating concentrations if possible, some protein targets can not be produced in sufficient amounts. In such cases, sub-saturating concentrations of protein targets were used successfully, due to the high sensitivity of the magnetic particle ELISA (data not shown).

**Example 15: High-throughput selection and screening of binding partners**

Phage display libraries (e.g. human scFv antibody fragment libraries, Tomlinson, unpublished) were screened for binders to various protein targets (see above). Phage titres were recorded after each round of selection to monitor the efficiency of phage amplification (data not shown). Using the magnetic particle phage ELISA, polyclonal mixtures of phage representing the unselected library and the results of every round of selection were screened for binding partners to the protein target used for this selection (e.g. UBI8, Fig. 5). Positive mixtures were cloned, and single colonies were rescreened by magnetic particle phage ELISA for binding to the same protein target (e.g. UBI8, Fig. 6).

## REFERENCES

Büssow, K., Cahill, D., Nietfeld, W., Bancroft, D., Scherzinger, E., Lehrach, H. & Walter, G. (1998). A method for global protein expression and antibody screening on high- density filters of an arrayed cDNA library. *Nucleic Acids Res* **26**(21), 5007-5008.

Crameri, R. & Blaser, K. (1995). Cloning allergens from *Aspergillus fumigatus*: the filamentous phage approach. *International Archives of Allergy & Immunology*. **107**, 460-1.

Crameri, R. & Suter, M. (1993). Display of biologically active proteins on the surface of filamentous phages: a cDNA cloning system for selection of functional gene products linked to the genetic information responsible for their production. *Gene*. **137**, 69-75.

Fisch, I., Kontermann, R. E., Finnern, R., Hartley, O., Solergonzalez, A. S., Griffiths, A. D. & Winter, G. (1996). A strategy of exon shuffling for making large peptide repertoires displayed on filamentous bacteriophage. *Proceedings of the National Academy of Sciences of the United States of America*. **93**, 7761-7766.

Griffiths, A. D., Williams, S. C., Hartley, O., Tomlinson, I. M., Waterhouse, P., Crosby, W. L., Kontermann, R., Jones, P. T., Low, N. M., Allison, T. J., Prospero, T., Hoogenboom, H. R., Nissim, A., Cox, J. P. L., Harrison, J. L., Zaccolo, M., Gherardi, E. & Winter, G. (1994). Isolation of high affinity human antibodies directly from large synthetic repertoires. *EMBO J*. **13**, 3245-3260.

Harrison, J. L., Williams, S. C., Winter, G. & Nissim, A. (1996). Screening of phage antibody libraries. *Methods in Enzymology* **267**, 83-109.

Hawkins, R. E., Russell, S. J. & Winter, G. (1992). Selection of phage antibodies by binding affinity: mimicking affinity maturation. *J. Mol. Biol.* **226**, 889-896.

Hefta, L. J., Wu, A. M., Neumaier, M. & Shively, J. E. (1996). Measuring antibody affinity using biosensors. In *Antibody Engineering, A Practical Approach* (McCafferty, J., Hoogenboom, H. R. & Chiswell, D. J., eds.), pp. 119-145. IRL Press, Oxford.

Kala, M., Bajaj, K. & Sinha, S. (1997). Magnetic bead enzyme-linked immunosorbent assay (ELISA) detects antigen-specific binding by phage-displayed scFv antibodies that are not detected with conventional ELISA. *Anal. Biochem.* **254**, 263-266.

Kay, B. K.; Winter, J.; McCafferty, J. Phage Display of Peptides and Proteins. A Laboratory Manual, Academic Press Inc: San Diego, CA, 1996.

Kirkham, P.M., Neri, D. & Winter, G. (1999). Towards the design of an antibody that recognises a given protein epitope. *J. Mol. Biol.* **285**(3), 909-915.

Lueking, A., Horn, M., Eickhoff, H., Bussow, K., Lehrach, H. & Walter, G. (1999). Protein microarrays for gene expression and antibody screening. *Anal. Biochem.* **270**(1), 103-111.

Low, N.M., P.H. Holliger, and G. Winter, *Mimicking somatic hypermutation: affinity maturation of antibodies displayed on bacteriophage using a bacterial mutator strain.* J Mol Biol, 1996. **260**(3): p. 359-68

McConnell, S.J., Dinh, T., Le, M.H., Brown, S.J., Becherer, K., Blumeyer, K., Kautzer, C., Axelrod, F. & Spinella, D.G. (1998). Isolation of erythropoietin receptor agonist peptides using evolved phage libraries. *Biol. Chem.* **379**(10), 1279-1286.

McConnell, S.J., Dinh, T., Le, M.H. & Spinella, D.G. (1999). Biopanning phage display libraries using magnetic beads vs. polystyrene plates. *Biotechniques* **26**(2), 208-210.

Mendelsohn, A.R. & Brent, R. (1999). Protein interaction methods - towards an endgame. *Science* **284**, 1948-1950.

Nissim, A., Hoogenboom, H. R., Tomlinson, I. M., Flynn, G., Midgley, C., Lane, D. & Winter, G. (1994). Antibody fragments from a 'single pot' phage display library as immunochemical reagents. *Embo Journal*. **13**, 692-8.

Schier, R., Balint, R.F., McCall, A., Apell, G., Larrick, J.W. & Marks, J.D. (1996a). Identification of functional and structural amino acid residues by parsimonious mutagenesis. *Gene* **169**(2), 147-155.

Schier, R., Bye, J., Apell, G., McCall, A., Adams, G.P., Malmqvist, M., Weiner, L.M. & Marks, J.D. (1996b). Isolation of high-affinity monomeric human anti-c-erbB-2 single chain Fv using affinity-driven selection. *J. Mol. Biol.* **255**(1), 28-43.

Smith, G. P. (1985). Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science*. **228**, 1315-1317.

Walter, G. & Tomlinson, I. M. (1996). Analysis of human antibody sequences. In *Antibody Engineering, A Practical Approach* (McCafferty, J., Hoogenboom, H. R. & Chiswell, D. J., eds.), pp. 119-145. IRL Press, Oxford.

Winter, G., Griffiths, A. D., Hawkins, R. E. & Hoogenboom, H. R. (1994). Making antibodies by phage display technology. *Annu. Rev. Immunol.* **12**, 433-455.